Development and characterization of primary cell culture from tissues of *Schizothorax plagiostomus*

PhD Synopsis

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1. Introduction

Fish provides the main source of high-quality protein to more than one billion people in the world. Most of the fishes are valued by the local people for their size and food value. The schizothoracine fish provide a good material to study patterns of evolutionary modifications in terms of their biogeographic distribution. The sub-family Schizothoracinae (Family: Cyprinidae), which includes snow trout *Schizothorax* spp. and several other genera are important for fisheries. Schizothoracines inhabit cold waters up to the altitude of 3,323 m, being present in rivers, streams and lakes; prefer temperature in the range of 8-22°C. The Genus *Schizothorax* is one of the most diversified and abundant group with 68 species around Central Asia, Himalayas, Pakistan, India and Tibetan Plateau (He and Chen, 2006).

Snow trout, *Schizothorax plagiostomus* is the most important food fish of different rivers,lakes and tributaries throughout Himalayas extending to parts of China, Afghanistan, Nepal, Bhutan, North East India, Kashmir and northern areas of Pakistan including river Panjkora Pakistan, Chen and Cao (2000) reported that *S. plagiostomus* can weigh upto 2.5 kg and attain 60 cm length. The fish S. plagiostomus commonly known as "Khont" is highly demanded food fish and has an edge over the exotic trout, because of its taste and good nutritional values. It is one of the most commercially important fish species of Kashmir Himalaya. It is an active fresh water fish found mostly in rivers. *S. plagiostomus* is widely distributed along the Himalayan foothills. However, recent observation have indicated drastic declines and change in the stock structure of this species in many areas of its range due to introduction of exotic species, damming, water impoundments and overfishing.

Fish comprise 48% of the known vertebrate species (Altman and Dittmer, 1972), which represents an enormous resource for the development of vertebrate cell and tissue models for use in biomedical sciences. Cell culture is the most important means available in life sciences to study the biological activities occurring inside cells of a living body and it refers to the *in-vitro* growth of cells taken from tissue of multicellular organisms. The cells are bathed in a culture medium containing essential nutrients and energy sources necessary for the cells survival.

Cell culture is one of the main tools used not only in molecular biological research but also for different applications including virus isolation and propagation, toxicity studies, production of high value human therapeutic fish proteins using recombinant DNA

technology and gene expression study. The fish cell culture would be used for surveillance of fish. Species specific fish cell cultures are required to isolate viral pathogens and their propagation for developing diagnostics and vaccines. A diversity of questions can be addressed with fish cell cultures. These include applications in biomedical research (Wafer *et al*. 2017), toxicology (Babich *et al.* 1986) and basic fish research (Smagghe *et al.* 2009), viral isolation (Chang *et al.* 2001; Kang *et al.* 2003; Luque *et al.* 2014; Wang *et al.* 2015) and, gene regulation, expression and gene transfer (Levy *et al.* 2000).

2. Research gaps

Since the past data pertaining to the cell culture of *S.plagiostomus* is not available so, this study is first of its kind and will focus on the following. Cell cultures prepared from fishes can provide a significant contribution to the growth of aquaculture. The scientific knowledge gained through the cell culture system can be utilized formanipulating the whole organism to enhance its usefulness for aquaculture. A cell-based aqua food production system utilizing cells in place of whole fish could also lead to greater preservation of the aquatic environments. Fish cell culture offers several advantages over mammalian cell culture in terms of adaptation to a broad range of temperature, higher tolerance to hypoxia, easier maintenance of cell culture for longer periods. The key areas of aquaculture like fish health, disease diagnosis, safety, and nutritional aspects challenging aquaculture production can be studied using fish cell lines without scarifying whole live fish. Cell lines would facilitate *in-vitro* research for developing climate resilient and sustainable aquaculture systems to minimize the key challenges and provide nutritional security to the world population.

3. Review of Literature

The development of fish cell lines has got momentum from previous efforts at Central Institute of Freshwater Aquaculture, (CIFA), Bhubaneswar in the early eighties and others attempt to develop cell line from *Cirrhinus mrigala* (Sathe *et al.,* 1995) and *Tor putitora* (Lakra and Bhonde, 2006).

3.1 Work done in India

India's first marine fish cell line was developed from the kidney of seabass, *Lates calcarifer* (Hameed *et al.* 2006), and subsequently cell lines from different species of fish from fresh water, marine and brackish water habitats were developed for different applications (Parameswaran *et al.* 2006, 2007). Department of Biotechnology, Govt. of India has played a pivotal role in expanding fish cell culture research in India. As a result, more than 750 fish

cell lines have been developed by different research groups in the country. Fish cell lines have been developed from many tissues such as ovary, fin, spleen, swim bladder, heart, liver, eye muscle, brain, and skin. Development of cell lines have been reported from important fish species like *Etroplus suratensis*, *Clarias batrachus* (Babu *et al.* 2011), *Schizothorax richardsonii* (Goswami *et al.* 2013), *Catla catla* (Chaudhuri *et al.* 2014), *Channa striatus*, *Puntius chelynoides* (Goswami *et al*. 2014) and *Wallago attu* (Dubey *et al.* 2014).

3.2 Work done in abroad

Hong, *et al*. (2004) developed spermatogonial cell line from the adult testis of Medaka (*Oryzias latipes*) and recapitulation of spermatogenesis from this cell line lead to the in vitro production of sperm without the presence of any somatic cells. They further characterized the identity of the cell line by transfecting them with either of the constructs pVegfp and pOct4gfp which give rise to GEP positive cells and also they analyzed the transcripts of the germ cells markers such as vasa, piwi, dazl and C-kit. The developed cell line transcribed all the four genesand thus confirmed the origin of the male germ cells.

Khamiss and Hashem (2012) while studying the developing cell culture system from Nile Tilapia using three culture media – L-15, MEM and RPMI reported that a higher cell attachment leading to cell proliferation was obtained using L-15 supplements with 20% FBS ascompared to MEM and RPMI that resulted in no cell development and cell proliferation.

Vo, *et al*. (2015) developed fibroblast- like WE cfin11f cell line from a walleye caudal fin and used to study the intersection of thermo-biology of walleye, Sander vitreus (Mitchill), with the thermal requirements for replication of viral haemorrhagic septicaemia virus (VHSV)IVb. WEcfin11f proliferated from 10 to 32ºC and endured as a monolayer for atleast a week at 1-34ºC. WEcfin11f adopted an epithelial shape and did not proliferate at 4ºC. Adding VHSVIVb to cultures at 4ºC and 14ºC but not 26ºC led to cytopathic effects (CPE) and virus production.

Yeh, *et al*. (2018) Established a continuous cell line ARB8 from the brain tissues of *Aequidens rivulantus*(Gunther) and its growth characteristics, transcriptions and susceptibility to fish viruses- including CSV, MEIPNV, GNNV, GSIV, KHV, HVA and MEPyV were examined. ARB8 cells showed epithelioid morphology and were passaged > 80 times in L-15 medium containing 5%-15% foetal bovine serum. The cells were found highly susceptible to CSV,MEIPNV, GSIV and RSIV and showed the typical cytopathic effect (CPE). However, the cells were resistant to GNNV, KHV, HVA and MEPyV because no significant CPE was noted after infection. The results revealed that the neural progenitor

cell line ARB8 can potentially serve as a tool investigating fish viruses and isolating new viruses in ornamental cichlid fishes.

Cell lines from different organs of trout have been developed by different workers in 1962 (Wolf and Quimby, 1962). A continuous cell line designated as RTgill-W1 has been established from gill tissue of rainbow trout (Bols *et al.* 1994). A permanent cell line has been developed from liver tissue of rainbow trout by the method of proteolytic dissociation using Leibovitz's L-15 medium supplemented with 5% fetal bovine serum (FBS) (Lee *et al.* 1993). The spleen cell line developed from rainbow trout by Ganassin and Bols (1996) was found to be very useful to study different functions of monocytes/macrophages and their response to different pathogens (Tafalla *et al.* 2008). Flano *et al.* (1998) described different types of splenic cultures established from explants of spleen of rainbow trout and found that they were susceptible to VHS virus. Five single clone lines have been developed from pituitary gland of adult rainbow trout and characterized, and *in-vitro* secretion of pituitary hormones was reported(Chen *et al.* 2010). Kawano *et al.* (2011) have established a continuous cell line from intestinal epithelial cells and this cell line should be a useful *in-vitro* tool to study the problems related to nutrition and gastrointestinal health in fish. Heart endothelial cell line was established from rainbow trout by immortalizing heart cells with recombinant retroviruses that transduced polyoma T antigen (Luque *et al.* 2014). A three-dimensional (3D) *in-vitro* experimental systemhas been developed from spheroid culture of the liver cell line of rainbow trout and it was found that the liver cells were viable and metabolically efficient (Lammel *et al.* 2019). In the present study, two novel cell lines were developed from the kidney and eye of rainbow trout and characterized, and applied for virological studies.

4. Objectives of the study

1. To Establish a primary cell culture from *Schizothorax plagiostomus*
2. To characterize the established primary cell culture
3. To conduct susceptibility testing of primary cell culture

5. Methodology

5.1. To establish a primary cell culture from *Schizothorax plagiostomus*

5.1.1. Collection, transportation and maintenance of fish

Fingerlings of 5-10 gm body weight of snow trout, *S. plagiostomus* will be collected from local fishermen. Live fish will be brought to the laboratory in a specially fabricated thermocol box of 5 ltr capacity with continuous aeration at 10 to 15°C. The fish will be brought to the

laboratory immediately. In the laboratory, the fish will be acclimatized for 24 h at 15°C in sterile fresh watercontaining kanamycin (1000 µg/ml) with continuous aeration.

5.1.2. Development of primary cell culture and sub-culturing. The individual fish will be transferred to fresh tank containing sterile aerated fresh water with kanamycin at the concentration of 500 µg/ml at 15 ± 2°C. The fish will be anesthetized by dipping into ice-cold water and the surface sterilization will be done using 70% ethanol followed by wiping using sterile phosphate buffered saline (PBS). Different organs will be dissected out aseptically, washed four times in Leibovitz's L-15 (GIBCO) medium containing antimicrobial agents (kanamycin [500 µg/ml] and fungizone [2.5 µg/ml]), and placed separately in sterile petri platesalong with medium. Each organ will be minced into small fragments of less than 1 mm3 in size.The tissue fragments will be placed in 25 cm2 flasks containing 5 ml of complete growth medium (Leibovitz's L-15 medium supplemented with 15% foetal bovine serum [FBS]), kanamycin (100 µg/ml) and fungizone (2.5 µg/ml). The flasks will be incubated at 16 ◦C in a normal atmospheric incubator. Cell culture flasks will be observed daily, and half of the medium will be replaced with fresh medium at intervals of 48 h. After 15 days, newly formed cells from tissue explant will form a monolayer. The monolayer of cells will be sub-cultured at a ratio of 1:2 to 1:3 following the standard trypsinization method using trypsin-EDTA solution (trypsin 0.25%, EDTA 0.2%) in phosphate buffered saline.

5.3 Characterization of established primary cell culture

Karyotyping: Chromosomal number will be determined using the method described by Freshney (1994). The cells will be treated with 0.04% colchicine and removed from the flask surface, and centrifuged at 500 × g for 5 min. The cell pellet will be gently suspended in 0.027 M KCl and incubated at 16 ◦C for 30 min. Cells will be then re-centrifuged again at 500 ×g for 5 min. Freshly prepared cold methanol-acetic acid (3,1) fixative will be added slowly to fix the cells in the pellet. These fixed cells will be then washed three times with fresh fixative, and thenre-suspended in a small amount of fixative. The cell suspension will be dropped onto glass slides, air dried and stained with 5% Giemsa (pH 6.8) for 15–20 min. Chromosome count will be carriedout in metaphase plates of fixed cells.

5.4 To conduct susceptibility testing of primary cell culture

In-vitro testing of primary cell culture will be carried out to study the effects of environmental toxicants or pharmaceutical drug on the cultured cells. Various concentrations of the test toxicant or drug will be used and thereafter the cytopathic effect and cellular changes will be correlated with each concentration gradient. These results will serve as a baseline data in an *in-vitro* cell culture model. In addition the developed primary cell culture

also offers application, to conduct viral susceptibility testing, virus isolation and characterization.

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